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Ultrastructural localization of tumour necrosis factor-alpha

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Summary

The application of an antibody against tumour necrosis factor-alpha (TNF) to thin sections of plastic-embedded mouse tissue has identified sites of TNF activity in normal and endotoxin-treated C₃H/HeN mice. Prior to endotoxin treatment, TNF was observed in the secretory granules of the antibacterial Paneth cell and one type of crypt endocrine cell. Four hours after endotoxin treatment, these two types of intestinal cell were found to have degranulated. In addition, endotoxin treatment resulted in the appearance of TNF in the secretory granules of all eosinophils, neutrophils and monocytes in the bone marrow, spleen, lung and the proximal intestine. TNF was also observed in the internal elastic lamina (IEL) of arterioles. These results suggest that the process of TNF induction specifically targets the immune system and the vasculature. An invasive stimulus, such as circulating endotoxin, can provoke the immune cells to be armed with TNF. That same stimulus may cause arteriole smooth muscle cells to secrete TNF. TNF secretion in the presence of arteriole smooth muscle cells may play a role in the adjustment of arteriole tone. In the venules, TNF may be responsible for platelet and neutrophil accumulation which leads to embolism formation.

Introduction

The relationship between endotoxin and septic shock with tissue injury has been well documented (Tracey *et al.*, 1986; Rothstein & Schreiber, 1988; Havell, 1989; Natanson *et al.*, 1989; Hinshaw *et al.*, 1990; Wakabayashi *et al.*, 1991). It has been suggested by Natanson and others, that endotoxin is not a requirement for septic shock since microorganisms, with or without endotoxin, can provoke a similar vascular response. It was thought that the invading microorganisms might be able to induce the expression of endogenous host mediators to target the vasculature and the immune system. The over-expression of these factors would ultimately cause the dysfunction of the target tissues. The cytokine tumour necrosis factor-alpha (TNF) (cachectin), has been considered to be one of the host factors involved. An invasive stimulus, such as sepsis, initiates TNF synthesis. Excessive secretion of TNF leads to the condition of cachexia which is manifested by shock, multiple organ failure, wasting and death (Beutler & Cerami, 1986).

To better understand the mechanism by which an invasive stimulus targets the vasculature and the immune

system, we have applied immunocytochemical techniques to localize TNF in tissues from normal and endotoxin-treated mice. In normal mice, we found the presence of TNF in the secretory granules of Paneth cells and endocrine cells in the crypts of Lieberkuhn of the proximal small intestine. Endotoxin treatment resulted in the appearance of TNF in the secretory granules of all neutrophils, eosinophils and monocytes examined. TNF was also localized in the internal elastic lamina of arterioles. The relationship between the site of TNF localization and the manifestation of tissue injury will be discussed.

Materials and methods

Animals

C3H/HeN female mice of about 20 g in body-weight were purchased from Charles River Laboratories (Raleigh, NC). Mice were maintained in Micro-Isolator cages (Lab Products, Maryland, IL) and maintained on commercial rodent chow and acidified water (pH 2.5) *ad libitum* in a facility accredited by the American Association for Accreditation of Laboratory Animal Care. All animals were quarantined on arrival and tested for

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Pseudomonas. Only healthy mice were released for experimentation. All animal protocols were pre-approved by an in-house Animal Care and Use Committee.

Endotoxin

Lipopolysaccharide derived from *Escherichia coli* (number 437635, Calbiochem Corp., San Diego, CA) was diluted to a concentration of $50 \mu\text{g ml}^{-1}$ with sterile saline, and 0.1 ml (5 μg) was injected into the lateral tail vein of each mouse. Four hours later, mice were killed by cervical dislocation and specimens removed and fixed. Specimens obtained from uninjected mice were used as controls.

Specimen preparation

Specimens were obtained from the proximal small intestine, spleen, bone marrow and lung and were immediately immersion-fixed in 1% paraformaldehyde (J. T. Baker Chemical, Phillipsburg, NJ) in a 100 mM cacodylate buffer (pH 7.3) containing 4 mM MgCl_2 . Tissue specimens were dehydrated in a graded series of methanol and embedded in L.R. White acrylic resin (The London Resin Co. Ltd, England) at 38°C in gelatin capsules. Thick sections were stained with a mixture of Methylene Blue (1%) and Azure II (1%) and photographed with a Zeiss Ultraphot microscope. Thin sections after cytochemistry were stained with 1% uranyl acetate in water and photographed with an Hitachi 7000 electron microscope. Because paraformaldehyde fixative has the disadvantage of not preserving cellular fine structure as well as glutaraldehyde does, attempts were made to perform studies using half-strength Karnovsky's fixative (a glutaraldehyde-containing fixative). Although it improved the general tissue appearance, this fixative also eliminated all binding of TNF antibody in the immunocytochemical assay. Washing tissue overnight to remove excess glutaraldehyde did not improve antibody binding.

Because of this limitation, paraformaldehyde was used as the fixative agent.

Antibody labelling

All thin sections were first floated on 20% normal whole goat serum (number 5006-1380 Cappel, Organon Teknika Corp., West Chester, PA). Without rinsing, sections were then floated overnight at room temperature on a 1:5 dilution of anti-TNF (number 654300 Calbiochem Corp., Mouse, Rabbit, IgG fraction), in antibody diluent. The antibody diluent was composed of 20 mM HEPES (*N*-2-hydroxyethylpiperazine-*N'*-2-ethansulphonic acid), 10 mM EGTA (ethyleneglycol-*bis*-(β -aminoethyl ether) *N,N,N',N'*-tetraacetic acid), 1% Tween 20, 1% bovine serum albumin, sodium chloride 0.43 M in deionized water. Control specimens were incubated overnight on a 1:100 dilution of normal rabbit serum (number 5012-1380 Cappel, Organon Teknika Corp., West Chester, PA) in antibody diluent. The following morning the specimens were rinsed with five changes of antibody diluent and then floated on a 1:10 dilution of 10 nm gold-conjugated goat anti-rabbit immunoglobulin G (number G-3766 Sigma, St Louis, MO) in antibody diluent for 1 h.

Results

Localization of TNF in specimens from normal mice

In normal (non-endotoxin-treated) mice, TNF label was found only in the granules of the Paneth cell and the endocrine cell in the intestinal crypt. Many granule-bearing cells, as a result of paraformaldehyde fixation, appeared to have been activated. This can be seen in the light microscopic image (Fig. 1). In Fig. 1A, the crypt region was sectioned longitudinally and its base was seen



Fig. 1. Light-microscopic images of the crypts of Lieberkuhn in the proximal small intestine of the mouse. Goblet cells (g) have secreted while the Paneth cells (p) are partially secreted. Endocrine cells (e) can be identified by their low optical density. Mast cells (mc) can be seen in the connective tissue. The lumen (l) is centrally located in each crypt. (A) Longitudinal section of the intestine containing a lymphatic duct (*). (B) Cross-section of the crypt region. $\times 1000$.

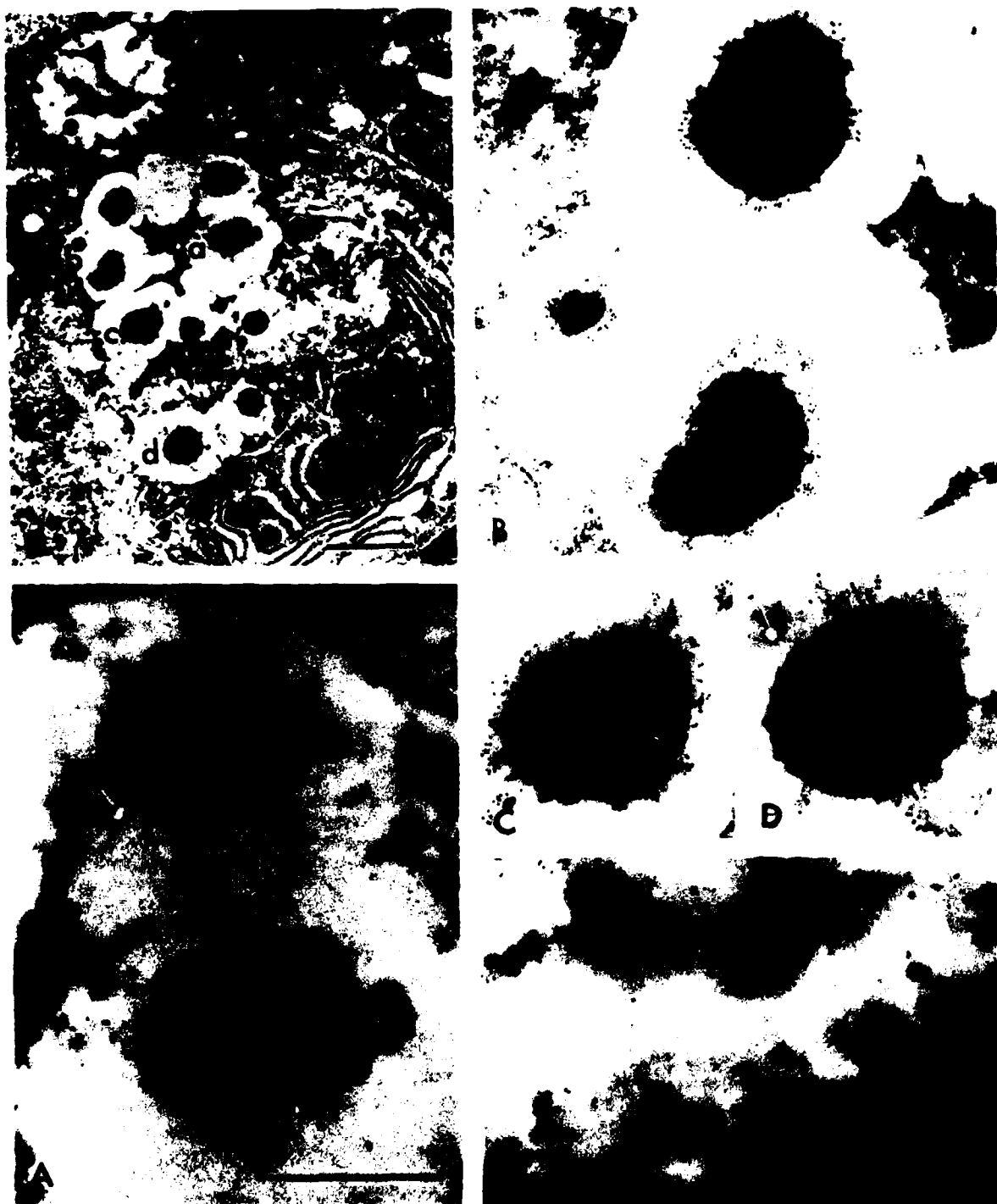


Fig. 2. TNF localization in a Paneth cell. TNF label was observed in the granules (a-d) and in the crypt lumen (e). Low magnification image $\times 5075$. Bar = $3\ \mu\text{m}$. (A-E) High-magnification images of areas marked (a) through (e) in the Paneth cell. The apparent bubbles (arrowheads) at the periphery of the activated granules are vesicles, and reflect the phospholipid content of the granules. High magnification $\times 27\,750$. Bar = $1\ \mu\text{m}$.

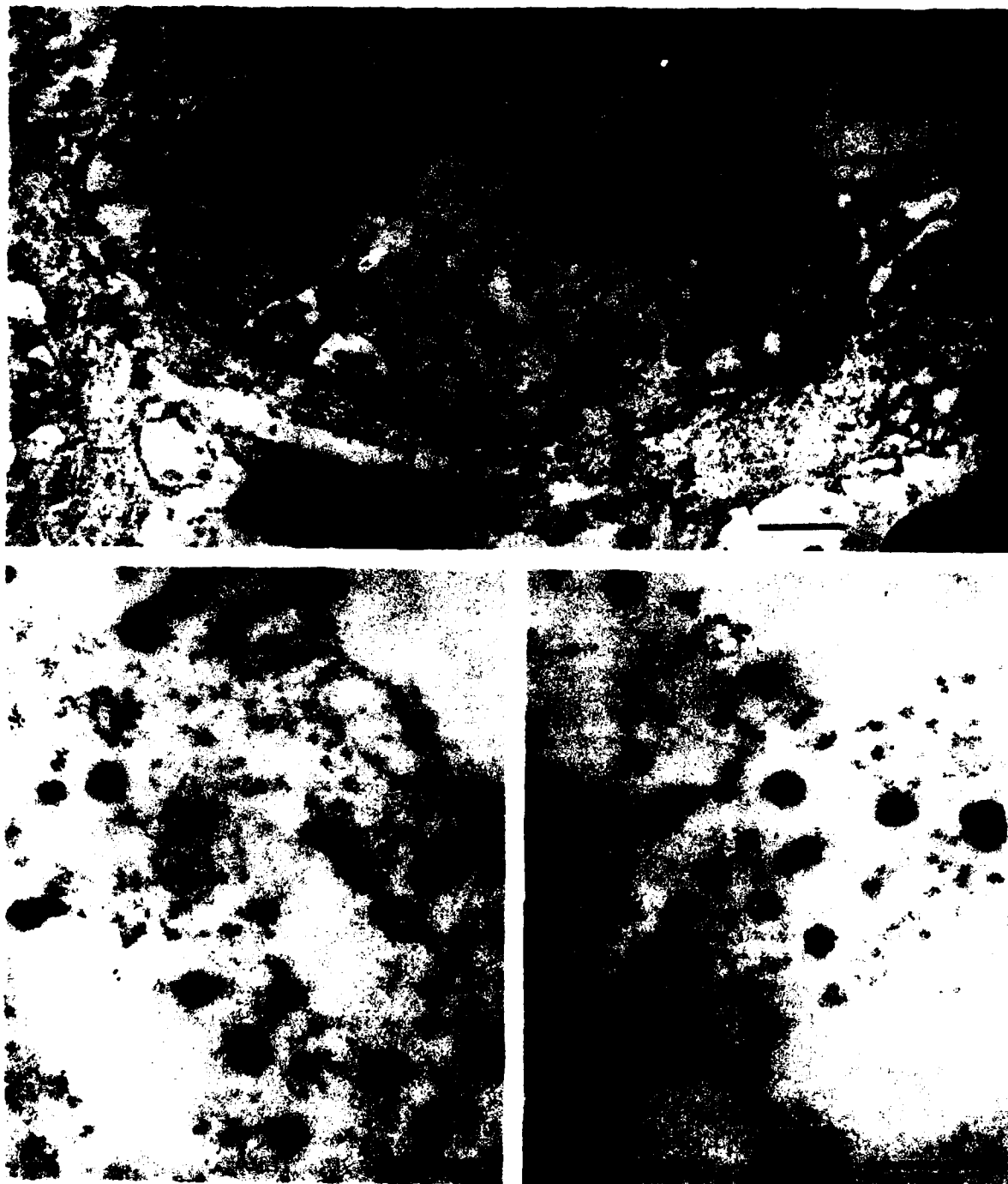


Fig. 3. Localization of TNF in the secretory granules of an intestinal endocrine cell. A secreting Paneth cell (p) in the upper right corner, a second endocrine cell (e) in the upper left corner, and a circulating red blood cell (rbc) in a capillary in the lower right corner are also shown. Low-magnification image = $\times 7350$. Bar = $2\ \mu\text{m}$. (A, B) High-magnification images of areas marked (a) and (b) in the endocrine cell. High magnification = $\times 38\ 500$. Bar = $0.5\ \mu\text{m}$.

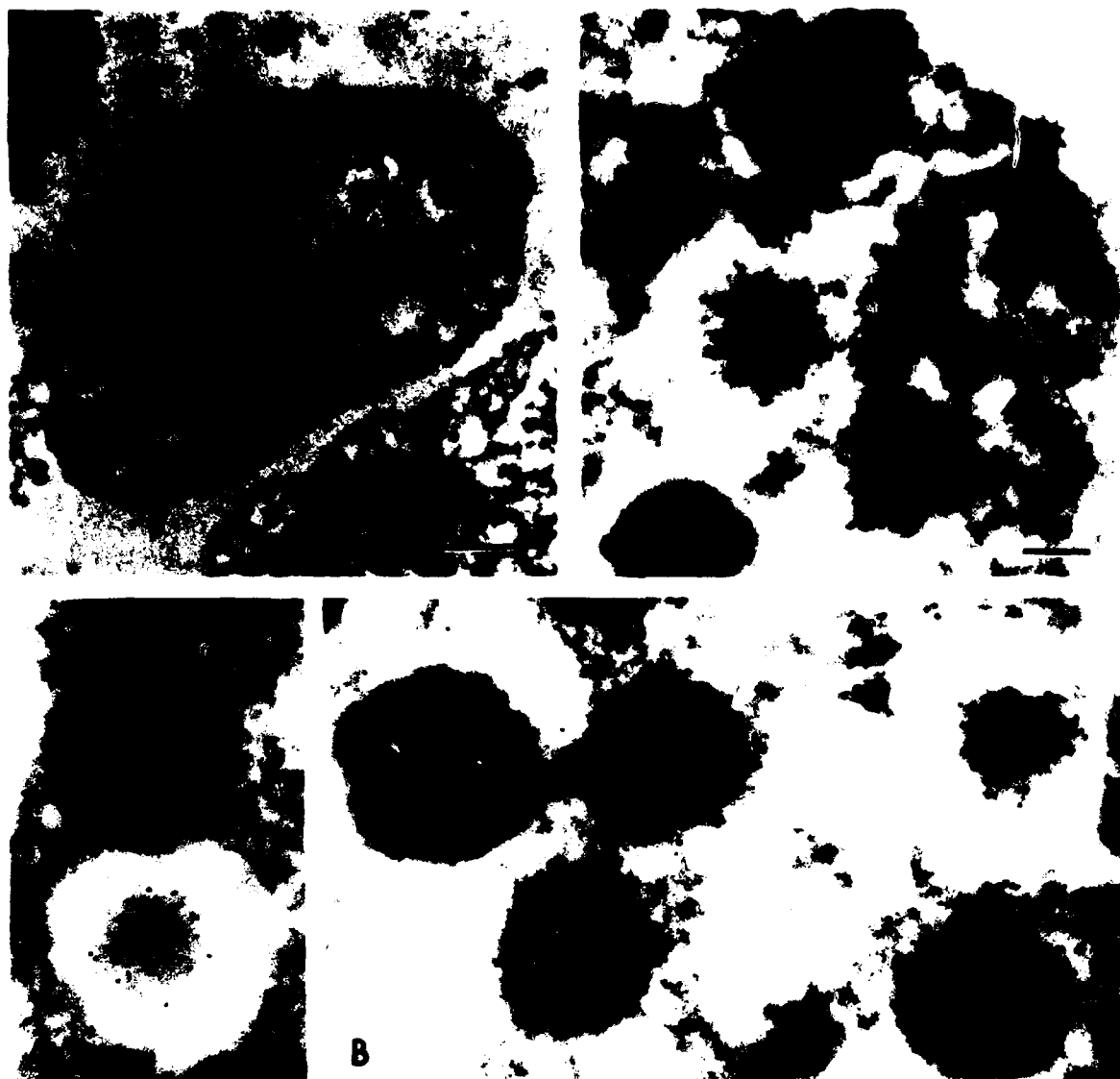


Fig. 4. Localization of TNF in the secretory granules of an eosinophil in the bone marrow after endotoxin administration. TNF label was observed in the secretory granules (a-c). Low magnification image of cell $\times 11,900$ Bar = $1\ \mu\text{m}$. A-C High magnification images of areas marked a-c in the eosinophil. High magnification $\times 51,000$ Bar = $0.2\ \mu\text{m}$.

to be occupied by Paneth cells and an unidentified endocrine cell. An endocrine cell with its cell body facing the submucosa and with a finger-like cytoplasmic extension touching the lumen was also seen (Fig. 1B). The connective tissue beneath the crypts is rich in lymphatic ducts and vasculature. The cross section of the crypts (Fig. 1B) also showed a large number of granule-bearing secretory cells.

The immunocytochemical localization of TNF in a typical Paneth cell is shown in Fig. 2. The activated granules were heavily labelled with anti-TNF, especially in the peripheral regions of the granules where their

matrices had become unravelled and dispersed. The inner domains of the granules which remained condensed appeared to have less TNF labelling (Fig. 2A-D). This may occur because these compact hydrophobic areas are less accessible to antibody penetration. The surface of the luminal mucosa contained TNF as a result of Paneth cell secretion (Fig. 2E).

TNF was also observed in association with the granules of the endocrine cells in the crypt (Fig. 3). These cells are normally located in areas adjacent to Paneth cells. The part of the cell body which contains the granules is usually located near the serosa and centrifugally from the

intestinal lumen aperture. These cells sample the luminal environment (Fig. 1B) and they release their granules near the basement membrane in response to stimuli arising in the lumen. They were easily identified by light- or electron-microscopy due to the low optical and electron density of their nucleus and cytoplasm.

Localization of TNF in specimens from endotoxin-treated mice

Endotoxin treatment resulted in the extensive degranulation of both the Paneth cells and the endocrine cells. Furthermore, endotoxin treatment also resulted in the appearance of TNF in the secretory granules of all eosinophils, monocytes and neutrophils in all tissues examined. Figures 4–6 show examples of labelled cells. In Fig. 4, an eosinophil in the bone marrow, after host endotoxin treatment, showed intense TNF activity in the secretory granules. TNF was also localized in bone marrow monocyte granules, as shown in Fig. 5. Several neutrophils in an intestinal venule were seen with light microscopy (Fig. 6A). One of these neutrophils (arrow), when examined with the electron microscope (Fig. 6B), exhibited cytoplasmic granules that contained TNF (Fig. 6, C–D). Circulating neutrophils of endotoxin-treated animals contained fewer granules than untreated controls, indicating that these cells had secreted as a result of endotoxin treatment. A large accumulation of platelets was seen adjacent to the neutrophils in the venule (Fig. 6A); within this accumulation there were amorphous materials that revealed a TNF content with high magnification (Fig. 6E). The platelets themselves did not exhibit TNF activity while the neighbouring neutrophils did. The neutrophils were assumed to be the source of this secreted TNF.

TNF was also localized in the internal elastic lamina (IEL) of arterioles in the bone marrow (Fig. 7). The images in Fig. 7 represent longitudinal sections through the lumen of an arteriole. The specific binding of TNF antibodies to the IEL was obvious. However, the origin of this TNF was not apparent because no significant localization of TNF was observed within either the adjacent endothelial cells or the underlying smooth muscle cells. Since the TNF label was seen in the elastin surrounding each smooth muscle cell in addition to the large ribbon which comprised the IEL, it can be assumed that the endotoxin-induced TNF might have been secreted by the smooth muscle cells with the elastin.

Specimens treated with normal pre-immune serum as control did not exhibit gold labelling.

Discussion

The role of TNF in terms of the immune function has been difficult to assign due to the many apparently opposing aspects of its actions. It has been implicated in derangement of host physiology as manifested by shock, wasting, multiple organ failure and death (Beutler & Cerami, 1986; Tracey *et al.*, 1986; Rothstein & Schreiber, 1988; Varani *et al.*, 1988; Luedke & Humes, 1989; Natanson *et al.*, 1989; Hinshaw *et al.*, 1990). Our observation of the sites of TNF localization has led us to believe that TNF may serve as a 'signal' to the host of an invasive trauma or threat. In this study, that threat was the presence of circulating endotoxin which was perceived by the host as a systemic bacterial infection. The host responded to this threat by inducing the synthesis and the release of TNF by cells of the immune system and vasculature.

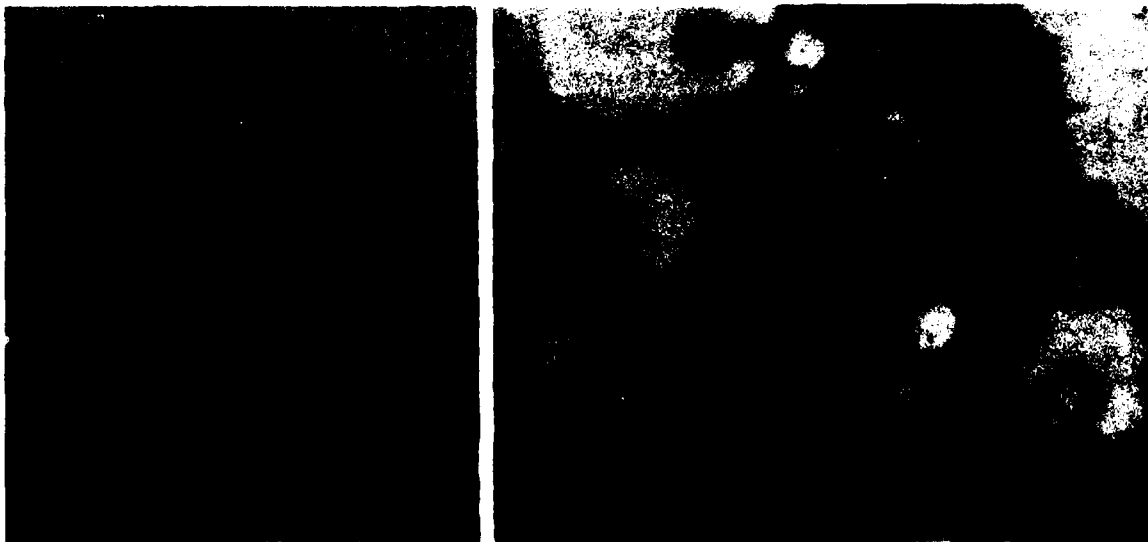


Fig. 5. Localization of TNF in the secretory granules of a monocyte in the bone marrow. On the left is the low-magnification image of the monocyte at $\times 8575$. Bar = 2 μm . On the right is the high-magnification image of the area marked with arrows in the monocyte. $\times 34\,500$. Bar = 0.5 μm .



Fig. 6. The intestinal crypt area 4 hours after endotoxin treatment. (A) Light micrograph. Paneth and endocrine cells have totally secreted their granules (*). The close proximity of the crypts to a lymphatic duct (ld), an arteriole (a) and a venule (v) can be seen. Neutrophils (n) in the venule are in association with an accumulation of platelets (p) in what may represent early embolism formation. A mast cell (mc) is seen in the connective tissue. Light micrograph magnification: $\times 1000$. (B) Electron micrograph of the neutrophil indicated with an arrow in the light micrograph. (p) is a platelet. Areas indicated by stars (*) in (B) are seen in high magnification in insets where the granules show label for TNF. Magnification for (B): $\times 5000$. Bar: $2 \mu\text{m}$. (C) Electron micrograph of area adjacent to platelet (p) shows an amorphous material labelled for TNF (arrowheads). Magnification for (C) and the insets in (B): $\times 50250$. Bar: $0.5 \mu\text{m}$.



Fig. 7. Localization of TNF in the internal elastic lamina of a bone marrow arteriole. (A) and (B) represent low magnification images of longitudinal sections showing the lumen (l), endothelial cells (ec), smooth muscle cells (sm), and the internal elastic lamina (arrowheads) of an arteriole. $\times 8550$. Bars = $1\ \mu\text{m}$. The inset represents a high magnification image of the elastin (e) shown in A while C represents the elastin (e) shown in B. High magnification. $\times 52,500$. Bars = $0.5\ \mu\text{m}$.

The Paneth cell and the intestinal endocrine cell reside within the crypts of Lieberkuhn. These crypts are pockets in the gut wall where bacteria, including endotoxin-producing Gram-negative bacteria, are normally present (Fig. 1). Our observations indicate that the chronic presence of bacteria in the gut induced TNF synthesis in the Paneth cell and the endocrine cell of normal, non-endotoxin-treated, animals (Figs 2 and 3). The administration of exogenous endotoxin caused these two types of cell to degranulate and release TNF further. The Paneth cell secretes TNF into the lumen and this may serve to signal the intestinal mucosa of a bacterial threat. The Paneth cell, which also secretes lysozyme and immunoglobulin A (Rodning *et al.*, 1976; Hauptman & Tomasi, 1976), may be an important component of the host intestinal immune defence system. Due to the strategic location of the endocrine cell granules, TNF secretion from these cells would be directed toward the vicinity of the vasculature and the lymphatic ducts in the connective tissues. This may serve to signal the circulating immune cells of a local presence of bacteria. This endocrine cell and the Paneth cell may play an important role in immune functions.

The injection of endotoxin into the general circulation induced the synthesis and the packaging of TNF in the secretory granules of all eosinophils, monocytes and neutrophils examined (Figs 4–6). The neutrophils and probably the monocyte, besides containing TNF in their granules, also exhibited fewer than a normal complement of granules. This might suggest that endotoxin had stimulated these cells to secrete their TNF into the circulation (Djeu *et al.*, 1990; Dubravec *et al.*, 1990). Neutrophils were often observed in the presence of large numbers of platelets within the venules (Fig. 6) in what appeared to be early embolism formation. The increased number of platelets in association with neutrophils was only observed in venules, and not in arterioles.

Following endotoxin treatment, TNF also appeared in the elastin surrounding each smooth muscle cell. This TNF was more apparent in the thick elastic ribbon, the IEL, which comprises the physical substrate for the attachment of endothelial cells in the arterioles (Fig. 7). The presence of TNF in the IEL and the release of TNF by neutrophils and other circulating immune cells in the arteriole, represents a potential exposure of the endothelial cells to TNF sources from both the lumen as well as the attachment matrix.

In our experiments we did not detect TNF labelling in the macrophage. This may be due to the fact that the maximal level of TNF induction in the macrophage usually occurs within one hour and declines rapidly thereafter (Chensue *et al.*, 1991). This narrow window of TNF production must have been missed in our four-hour time period between endotoxin injection and tissue fixation.

Rothstein & Schreiber (1988) have studied the endotoxin response in pathogen-free mice. They concluded that, for tissue injury to occur, four criteria needed to be met. These included endotoxin priming, TNF secretion,

neutrophil activation, and an unidentified 'cellular responsiveness'. In our experiment, we have also observed TNF secretion and neutrophil activation following endotoxin priming. Furthermore, we have also consistently observed a response by the vasculature. This vascular responsiveness to endotoxin includes neutrophil and platelet aggregation (embolism formation) in the venules and an accumulation of TNF in the arteriole wall, that may imply a change in the arteriole tone.

We propose that, once the 'signal' of an invasive threat is perceived by the host, circulating granulocytes and monocytes become armed with TNF. They circulate normally and will secrete their TNF only when and where they are challenged by a specific stimulus. This makes the release of TNF very site-specific and localized.

In our experiments, endotoxin was injected systemically into the circulation, causing cells of the immune system and vasculature to become armed with TNF. Since neutrophils specifically respond to bacteria, the systemic presence of endotoxin could potentially trigger all neutrophils to secrete their TNF throughout the circulation.

The stimulation of neutrophils is accompanied by the release of lipid mediators such as platelet activating factor (Sisson *et al.*, 1987; Warren *et al.*, 1990; Mozes *et al.*, 1991). In the venules, the endotoxin-triggered release of TNF and lipid mediators may be instrumental in causing the aggregation of platelets and neutrophils resulting in clot formation and venule occlusion.

The drastic drop in the mean arterial blood pressure associated with endotoxic shock has been linked to the release of lipid mediators such as prostaglandin E_2 (PGE_2), a well known vasodilator substance (Messina *et al.*, 1976; Kettelhut *et al.*, 1987; Mozes *et al.*, 1991). The localization of cyclo-oxygenase and PGE_2 in the secretory granule, and the localization of PGE_2 in the IEL of intestinal arterioles, have also been reported (Schmauder-Chock & Chock, 1989, 1992). It can be implied that TNF and lipid mediators may be responsible for the drop in arterial tone associated with septic shock. Our localization of TNF, and additionally, the previous localization of PGE_2 to the IEL, lead us to consider that the arterial smooth muscle may react to circulating stimuli with the production of vaso-active mediators such that they may self-regulate their own tonicity. This may be a means of regulating the local blood flow.

Venule occlusion may be an intrinsic mechanism of the vasculature to confine invasive bacteria to the capillary bed and prevent their distribution into the general circulation. Venule occlusion, when coupled with a drop in the arteriole tone, may serve to increase the capillary blood volume and to facilitate the influx of immune cells to combat the invasive bacteria. Therefore, in the micro-environments, these mechanisms serve to limit the scope of an invasive threat and facilitate the elimination of bacteria from the circulation. However, when they function systemically, they may induce shock, multiple organ failure, wasting and death.

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